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(54) Title: AEROSOL ADMINISTRATION OF INTERLEUKIN-2 LIPOSOMES

(57) Abstract: IL-2 liposome formulations suitable for aerosol delivery are described. Also described are methods of treating patients with immune deficiency conditions or chronic viral infections, such as hepatitis C, by the administration of such aerosolized IL-2 liposome formulations. The IL-2 liposome formulations can be self-administered by the patient. Also described are kits including the IL-2 liposome formulations.

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AEROSOL ADMINISTRATION OF INTERLEUKIN-2 LIPOSOMES

BACKGROUND OF THE INVENTION

IL-2 has been used for the treatment of cancer and infectious diseases, but has substantial systemic toxicity and a narrow therapeutic index. See Kaplan (1994) J. Chrom. B: Biomed. Appl. 62:315; Rosenberg et al. (1985) N. Engl. J. Med. 313:1485; Sznol et al. (1989) Cancer Treat. Rev. 16:29-38; Smith (1993) Blood 81:1414; Siegal and Puri (1991) J. Clin. Oncol. 9:694; and Mekhail et al. (2000) BioDrugs2000 14:299-318.9-14. And although parenteral high-dose IL-2 (e.g. >3 x10^6 IU/day) has been shown to have beneficial effects in patients with cancer, common variable immunodeficiency (CVID), and human immunodeficiency virus (HIV), use and acceptance has been limited by this toxicity, especially capillary leak and flu-like symptoms. In view of IL-2 toxicity when given by the parental or subcutaneous routes, newer means for the therapeutic delivery of IL-2 are clearly needed.

Liposomes are microscopic lipid vesicles absorbed into lymph nodes and other organs within the mononuclear phagocytic system (e.g. spleen, liver, lung). Liposomes may be designed to carry a variety of substances including proteins and cytokines. See Anderson et al. (1994) Cytokine 6:1-10. Liposomes containing IL-2 and human serum albumin as a carrier have been previously described in U.S. Patents Numbers 5,409,698, 5,650,152 and 5,773,006 for parenteral administration in the treatment of cancer and for co-administration as an immunoadjuvant with vaccine preparations.

Parenteral administration of liposomes containing IL-2 has been shown to activate the immune system with decreased toxicity. See Mbawuike et al. (1990) Vaccine 8:347. Such parenteral administration of IL-2 in liposomes achieved higher concentrations of IL-2 within tissues of the immune system including lung, liver, spleen, lymph nodes, and bone marrow compared to free IL-2. See Gause et al. (1993) Am. Soc. Clin. Onc. Proc. 12, 955; Anderson et al. (1992) Drug Devel. Res. 27:15-31; Anderson et al. (1992) J. Immunother 12:19-31; Khanna et al. (1996) Clin. Cancer Res. 2:721-34; and Khanna et al. (1997) Cancer 79:1409-1421. IL-2:human serum albumin (IL-2:HSA) liposomes have

been given intravenously to patients with metastatic cancer, inducing immune activation with minimal toxicity. See Khanna et al. (1997) J. Pharm. Pharmacol. 49:960-71.

Canine studies on aerosol delivery of IL-2 liposomes have demonstrated the biologic activity and safety of aerosols of IL-2 liposomes in normal dogs (Khanna et al. (1996) Clin. Cancer Res. 2:721-34 and Khanna et al. (1997) J. Pharm. Pharmacol. 49:960-71). Such treatment led to the regression of naturally occurring pulmonary metastases in dogs after one month of nebulized IL-2 liposome therapy (Khanna et al. (1997) Cancer 79:1409-1421). No significant toxicity was observed in a recent phase I clinical trial on the inhalation of IL-2 liposomes for the treatment of pulmonary metastases. See Skubitz and Anderson (2000) Anti-cancer Drugs 11:555-563.

SUMMARY OF THE INVENTION

The invention is based on the development of reliable and reproducible methods for the production of clinical grade IL-2 liposomes without albumin carrier protein suitable for aerosol use. These methods can be reproduced by any pharmacy with access to a laminar flow hood and familiar with the filling of vials for unit dosing. The invention is also based on the discovery that IL-2 liposomes can be administered by aerosol delivery to individuals with immune deficiency conditions and chronic viral infections, such as hepatitis C. Such aerosolized IL-2 liposomes can be self-administered by the patient. Patients with primary immune deficiency or chronic hepatitis C infection were treated with a non-albumin containing IL-2 liposome formulation by the aerosol route in limited duration, double blind, placebo control crossover studies. Results show that chronic self-administration of aerosol IL-2 liposomes is feasible in patients with chronic hepatitis C, has excellent patient acceptance and low toxicity and is effective in decreasing viral titers and viral loads.

In general, the invention features a method of treating patients with a chronic viral infection by administering IL-2 liposomes. The chronic viral infections treated include, but are not limited to hepatitis, including hepatitis B and hepatitis C, HIV and AIDS. The IL-2 liposomes can be administered by aerosol and can be self-administered. The treatment method of the invention can be administered as an adjuvant to other therapies,

including the administration of interferon α-2b and ribavirin (IFN/R) for chronic hepatitis C.

The invention also features a method of treating a patient suffering from of a bacterial infection, a viral infection, a fungal infection, a parasitic infection, or an immunodeficiency condition by administering aerosolized IL-2 liposomes. The immunodeficiency condition can be, but is not limited to, common variable immunodeficiency (CVID). The viral infection can be, but is not limited to, human immunodeficiency virus (HIV). The patient can also be suffering from complications of AIDS. The aerosolized IL-2 liposomes can be self-administered.

In another aspect, the invention features kits including sterile, lyophilized IL-2 liposomes. These kits can further include a label or package insert indicating that the lyophilized IL-2 liposomes are to be reconstituted and administered via nebulizer treatment. The IL-2 liposomes included in the kit can be albumin-free.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice and testing of the present invention, suitable methods and materials are described. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a FACS analysis of liposome size distribution. Figure 1A represents polystyrene beads 4.2 μm standard size. Figure 1C represents polystyrene beads 21.1 μm standard size. Figures 1B and 1D represent IL-2 liposomes compared to 4.2 μm

polystyrene beads C and 21.1 μm polystyrene beads, respectively. Note: >95% of liposomes are <5 microns.

Figure 2 represents bioactivity of the IL-2 liposomes as determined by ^{3}H-thymidine incorporation assay with the IL-2-dependent CTLL-2 cell line. Figure 2A is bioactivity of IL-2 liposomes in triplicate plates, compared to standard free rIL-2. Figure 2B is bioactivity of the IL-2 liposome pellet compared to the supernatant, in duplicate plates.

Figure 3 is a diagram of the protocol used for the administration of aerosol liposomes and schedule of the tests. Dashed areas indicate the time frame when aerosol liposomes (with IL-2 or empty, in a randomized order) were given. Washout indicates the time when patients did not receive any treatment, to avoid carryover effects from the first aerosol liposomes administered. Blood tests include serum Ig, lymphocyte proliferation (to mitogens and antigens), and lymphocyte surface markers. PFT is pulmonary function tests. CXR is chest radiograph. Pneumovax was administered only to patients with specific antibody deficiencies at the indicated times.

DETAILED DESCRIPTION

The current invention is based on the development of reliable and reproducible methods for the production of clinical grade IL-2 liposomes without albumin carrier protein suitable for aerosol use. These methods can be reproduced by any pharmacy with access to a laminar flow hood and familiar with the filling of vials for unit dosing. The current invention is also based on the observation that the aerosol delivery of liposomes containing IL-2 to individuals with chronic viral infections was effective in decreasing viral titers and viral loads. These results indicate that chronic self-administration of aerosol IL-2 liposomes is effective and has excellent patient acceptance and low toxicity.

LIPOSOME FORMULATIONS

The invention features liposome formulations comprising IL-2. The method of preparing the liposome formulations includes, but is not limited to, the method described in Example 1. Liposomes can also be prepared by any known methodology. Throughout this patent, all reported IL-2 units of biological activity are reported in International Units

(IU), which is defined as 15 x 10^6 units per mg IL-2 protein. The IL-2 of the liposome formulation includes all known forms of IL-2, including pharmaceutically acceptable analogues and derivatives thereof. For example, recombinant non-glycosylated human IL-2, obtained from Chiron (Proleukin, Aldesleukin; Emeryville, CA), can be used in the liposome formulation. Earlier formulations of IL-2 liposomes contained human serum albumin for historical reasons, due to the fact that natural sequence Hoffmann La Roche IL-2 (Nutley NJ) contained albumin. Currently available clinical grade IL-2 (Chiron, Emeryville CA) does not contain albumin. Because of recent FDA concerns about use of albumin in parenteral drugs, the following procedures synthesize clinical grade IL-2:DMPC liposomes that contain no human serum albumin.

In addition to IL-2, the liposome formulations of the current invention may contain additional agents. Such agents include, but are not limited to, additional cytokines that induce T-cell proliferation or that induce the generation of a Th1 T-cell population. Examples include the interferons (including IFNα, IFNα2b or IFNγ), IL-10 and tumor necrosis factor (TNF). Such agents can also include viral polypeptides, or antigenic fragments thereof. Such viral polypeptides can include, but are not limited to, the nucleocapsid C protein and the nonstructural HCV proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b) of the hepatitis virus. See Lohman et al. (1996) J. Hepatol. 24(2 Suppl):11-19. Such agents can also include nucleic acid analogues and/or protease inhibitors used in the treatment of retroviral infections. Examples of such antivirals includes, but are not limited to, ribavirin (R), azidovudine (AZT), 2',3'-dideoxyinosine (ddI), 3'-azido- 2', 3'-dideoxythymidine, acyclovir, 1, 3-dihydro-2-propoxy-methyquanine (gancyclovir), ribavirin, dideoxycytidine (ddC), lamivudine (3TC) and protease inhibitors, such as saquinovir and invirase. Other agents can include anti-bacterial agents (for example, pentamidines, trimethoprim-sulfamethoxazole, sulfonamides, penicillins, cephalosporins, aminoglycosides, tetracyclines, chloramphenicols), anti-fungal agents (for example, flucytosine, amphotericin B, fluconazole, griseofulvine), and anti-parasitic agents (for example, pyrimethamine, quinacrine, thiabendazole or levamisol). Any number of additional agents may be added in any combination thereof.

Ribavirin (R), an oral nucleoside analogue, has been tested in patients with chronic hepatitis C through placebo-controlled clinical trials. Although 20% to 40% of

patients had a biochemical response at the end of therapy, none had a virologic response. On the contrary, the combination of interferon and ribavirin in treating chronic hepatitis C was associated with a higher virologic response than IFN or ribavirin alone. The combination of IFN and ribavirin was associated with sustained virologic response in 47% of IFN-naïve patients, in 80% of those who had relapse after an end-of-treatment response to a previous course of IFN, and in 15% of those who had no response at all to a previous IFN treatment. See Dusheiko et al. (1996) J. Hepatol. 25:591-98; Di Bisceglie et al. (1994) J. Hepatol. 21:1109-12; Chemello et al. (1995) J. Hepatol. 23 Suppl 2:8-12; Schvarcz et al. (1995) J. Hepatol. 23 Suppl 2:17-21; and Brillanti et al. (1994) Gastroenterology 107:812-7. A recent meta-analysis of individual patient data in four European centers concluded that the efficacy of IFN and ribavirin combination therapy has a twofold- to threefold-enhanced efficacy over IFN alone. See Schalm et al. (1997) J Hepatol 26:961.

KITS/ARTICLES OF MANUFACTURE

IL-2 liposome formulations may be packaged as kits or articles of manufacture that contain one or more unit dosage forms. The article of manufacture typically comprises at least one container with a label. This container may, for example, comprise glass or plastic bottles or vials, metal or plastic foil and may also include unit dose blister packaging. The container holds a composition comprising an IL-2 liposome formulation. This formulation may be in lyophilized form. The IL-2 liposome formulation may include agents in addition to IL-2, such as those listed above. The IL-2 formulation may be free of human serum albumin as a carrier. A label or package insert indicating that the IL-2 formulation is to be administered in aerosol form may accompany the package. The directions may indicate that the IL-2 liposome formulation may be used alone or as an adjuvant to other therapies. The package may include additional agents to be administered separately from the aerosolized IL-2 liposome formulation. Examples of such agents include, but are not limited to, interferon (IFN), Ribavirin (R), IFN/R, pegylated interferon, intravenous Ig (IVIG), protease inhibitors, nucleic acid analogues, antibacterial agents, anti-fungal agents and anti-parasitic agent. For example, the kits may also include pegylated interferons (peginterferons) representing second-generation

interferons that have been developed mainly for the treatment of chronic HCV infection. Two companies simultaneously developed peginterferon forms of their existing nonpegylated interferons. Schering-Plough Pharmaceuticals developed peginterferon alpha-2b (Peg Intron), and F. Hoffmann-LaRoche Pharmaceuticals developed peginterferon alpha-2a (Pegasys) by chemically conjugating a polyethylene glycol molecule with interferon alpha-2b or interferon alpha-2a, respectively. Reports have been published recently on the safety and efficacy of peginterferon alpha-2a in patients with chronic HCV infection. See Zeuzem et al. (2000) N. Engl. J. Med. 343(23):1666-1672 and Heathcote et al. (2000) N. Engl. J. Med. 343(23):1673-1680. In the report by Zeuzem et al., 531 interferon-naïve patients with chronic HCV infection were randomly assigned to treatment with standard interferon alpha-2a (3 times weekly) or peginterferon alpha-2a (once weekly) for 48 weeks. Peginterferon alpha-2a was associated with a significantly greater sustained virologic response than was interferon alpha-2a (39% vs. 19%, $P = 0.001$). The rate of sustained virologic response among the patients receiving peginterferon alpha-2a monotherapy in this study is clearly comparable to historical figures associated with the combination therapy of standard interferon-α with ribavirin. The report by Heathcote et al. described the safety and treatment outcome of peginterferon alpha-2a in 271 patients with chronic HCV infection who had advanced fibrosis or cirrhosis. Similar to the study of Zeuzem, patients were randomly assigned to treatment with standard interferon alpha-2a or peginterferon alpha-2a for 48 weeks. Peginterferon alpha-2a, given at a dosage of 180 μg weekly, was associated with a significantly greater sustained virologic response than was interferon alpha-2a (30% vs. 8%, $P = 0.001$). In both studies, the frequency and severity of adverse effects in those receiving peginterferon alpha-2a were similar to those of patients receiving standard interferon alpha-2a.

The article of manufacture may include other materials desirable from a commercial or user standpoint. Such materials include, but are not limited to, saline for resuspending lyophilized IL-2 liposome formulations (e.g. bronkosaline), buffers, filters, needles, syringes, inhalers, nebulizers, supplies for inhalers and nebulizers and package inserts with instruction for use.

METHODS OF TREATMENT

Aerosolized IL-2 liposome formulations may be administered as a therapeutic agent to treat patients with immune deficiency or chronic viral infections, including hepatotrophic viral infections and hepatitis. Administration of aerosol IL-2 liposomes formulations includes administration by a health care provider and self-administration by the patient. Administration may be accomplished using a nebulizer. Aerosol treatments may take place repeatedly on any given day, for example, 1, 2, 3, 4 or 5 times per day. Aerosol treatments may take place every day or at less frequent intervals, for example, every other day or weekly. Aerosol treatments may continue for a short period of time, for example, for a time period of 1-5 weeks. Treatments may also continue long term, for example for a time period of 1-11 months, for 1-5 years or longer. Any dosage of IL-2 may be administered. Dosages to be administered include, but are not limited to, 0.1, 0.5, 1.0, 2, 2.5, 5.0 and 10 x 10^6 IU/day. IL-2 liposome formulations may be administered using dose escalation and/or induction therapy. Dose escalation refers to an incremental increase in the dose of IL-2 liposome formulation or its frequency of administration. Induction therapy refers to the practice of giving a higher-than-conventional dose early in the course of therapy, followed by a routine dosage regimen. The administration of aerosolized IL-2 liposome formulations to a patient may be as an adjuvant therapy, in addition to other therapies.

HEPATITIS VIRAL INFECTIONS

IL-2 liposone formulations can be administered for the treatment of chronic viral infections such as hepatitis. Five viruses (A, B, C, D, and E) have been identified as agents of acute or chronic hepatitis. The latest of these is an RNA virus cloned from sera of patients with non-A, non-B, non-C hepatitis and named "hepatitis G virus" (HGV). The list of human hepatitis viruses undoubtedly will continue to grow because some patients with hepatitis or cirrhosis have no markers for the currently known viruses. Among all hepatitis viruses, hepatitis B virus (HBV) and hepatitis C virus (HCV) are thought to be most significantly associated with the development of end-stage liver disease and hepatocellular carcinoma (HCC). Hepatitis A virus (HAV) and hepatitis E virus (HEV) are associated with self-limited acute hepatitis syndrome and are transmitted

by the orofecal route. Because of the self-limited nature of these two infections, treatment with interferon (IFN) has not been tested and, currently, is not recommended. IFNs are a group of low molecular weight proteins produced by leukocytes (IFN-α), fibroblasts (IFN-β), and T-lymphocytes (IFN-γ). These proteins have antiviral effects that seem to operate through several mechanisms, including inhibition of viral replication, inhibition of viral protein production, and prevention of the release of virions from infected cells. IFN-α was the first to undergo clinical trials and is the IFN used most extensively to treat viral hepatitis. More recently, consensus IFN (CIFN) has been generated synthetically by researchers at Amgen Corporation (Thousand Oaks, CA) and has been approved by the U.S. Federal Drug Administration (FDA) for the treatment of chronic hepatitis C infection. Reviewed in Zein (1998) Cytokines Cell. Mol. Ther. 4:229-41

HBV is a member of the family of viruses known as hepadnaviridae. These viruses share the characteristics of a partially double-stranded DNA surrounded by an outer lipoprotein envelope and an inner core composed of nucleocapsid proteins. After acute exposure, 90% of patients with HBV infections are asymptomatic or have mild symptoms (anorexia, nausea, and fatigue). Approximately 10% of patients are overtly ill and jaundiced. In 1% to 2% of acutely infected patients, HBV disease worsens over a period of 1 to 3 months and coagulopathy gradually occurs (liver failure). It has been estimated in prospective studies conducted in Europe that 5% to 10% of adults become chronic carriers after acute HBV infection. Although acute HBV infection is a benign disease in most cases, chronic HBV infection is a serious and often progressive liver disorder that affects more than 5% of the world's population. Approximately 300 million people are estimated to be chronic carriers of HBV, as manifested by the presence of circulating hepatitis B surface antigen (HBsAg). Over many years, chronic hepatitis can lead to cirrhosis or HCC. Tumor development occurs as a result of HBV DNA integration into the host hepatocyte genome. Prognosis of chronic infection is determined predominantly by the presence of active viral replication (serum positive for HBeAg and HBV DNA) and the degree of histologic liver damage. Chronic HBV carriers with active viral replication are at highest risk for the development of progressive disease, with 15% to 20% developing cirrhosis within 5 years after diagnosis. Furthermore, active HBV

replication is associated with increased risk of transmission by needlestick exposure or by the vertical route. Therefore, patients with chronic HBV serve as reservoirs for infection and constitute a potential public health threat. For these reasons, treating chronic HBV may potentially prevent the progression of liver disease and may slow the spread of infection. However, treating acute HBV is not recommended because it is a self-limited disease in most of those infected. See review by Zein (1998) Cytokines Cell. Mol. Ther. 4:229-41.

Since the discovery of HCV infection in 1989, considerable knowledge has been learned about its natural history. Acute hepatitis C generally is a benign disease. In the transfusion setting, where acute onset is best documented, 70% to 80% of cases are anicteric and asymptomatic. Documented fulminant hepatitis C is extremely rare. The significance of HCV infection resides in its tendency to become persistent and to induce chronic liver disease. Worldwide, hepatitis C accounts for more than 90% of post-transfusion chronic hepatitis and cirrhosis. Unlike HBV, HCV evolves into chronic disease associated with liver damage in more than 80% of cases. Furthermore, a strong association has been established between HCV infection and HCC, independently of the presence or absence of hepatitis B markers or other underlying liver disease. Also, it has been suggested that patients with anti-HCV-positive HCC have a worse prognosis than those with HBsAg-positive HCC or those with alcoholic liver disease-associated HCC. Currently, in the U.S., chronic HCV infection is the leading cause of end-stage liver disease requiring transplantation and is believed to be the agent responsible for most cases of post-transplantation hepatitis. Because of the high rate of chronicity following acute HCV, investigators have attempted to treat both acute and chronic HCV. Although the goal of treating acute infection is to prevent progression to chronic liver disease, treatment of chronic HCV is directed mainly at preventing complications (cirrhosis and HCC). See review by Zein (1998) Cytokines Cell. Mol. Ther. 4:229-41.

COMMON VARIABLE IMMUNE DEFICIENCY (CVID)

Common variable immune deficiency (CVID) is a heterogenous set primary immune deficiency manifested by marked reduction in serum immunoglobulin (Ig) levels and a decreased ability to mount specific humoral responses to antigens *in vivo*. See

Cunningham-Rundles (1989) J. Clin. Immunol. 9:22-32; Antall et al.(1999) J. Allergy Clin. Immunol. 103:637-41; and Rump et al. (1992) Clin. Exp. Immunol. 89:204-10.3. Standard treatment of CVID and other humoral immune deficiencies is monthly intravenous Ig (IVIG) replacement for life. Recent shortages of IVIG, cost, viral contamination of IVIG, and association of IVIG with serious side effects in some patients have prompted searches for alternative means to improve immune function. See Cunningham-Rundles et al. (1995) J. Interferon Cytokine Res. 15:269-76 and Cunningham-Rundles et al. (1992) Clin. Immunol. Immunopath. 64:46-56.

Some patients with CVID have been found to display aberrant interleukin 2 (IL-2) mRNA levels. See Rump et al. (1992) Clin. Exp. Immunol. 89:204-10 and Fisher et al. (1993) J. Allergy Clin. Immunol. 92:340-52. Proliferative responses of some CVID T cells in culture can be significantly improved by the addition of recombinant IL-2. See Kruger et al. (1984) J. Clin. Immunol. 4:295-303. In addition, IL-2 induces the secretion of Ig *in vitro*. See Nakagawa et al. (1987) J. Immunol. 138:795-801. PEG-IL-2 has been administered intravenously or subcutaneously to patients with CVID in an attempt to enhance *in vitro* T and B cell function (15-17). Most of these patients showed improvement of *in vitro* B cell function after PEG IL-2 as shown by increased *in vitro* total and antigen-specific Ig secretion. See Cunningham-Rundles et al. (1992) Clin. Immunol. Immunopath. 64:46-56. In addition, some patient showed enhanced T cell proliferation and normal IL-2 production *in vitro* after 12 weeks. See Cunningham-Rundles et al. (1995) J. Interferon Cytokine Res. 15:269-76. However, PEG-IL-2 failed to significantly increase serum IgM and IgG levels *in vivo* and the requirement for IVIG treatment was not significantly reduced during PEG IL-2 treatment.

Some individual CVID patients appeared to benefit from the IL-2 treatment and had a significant reduction in the number of severe infections. See Cunningham-Rundles et al. (1992) Clin. Immunol. Immunopath. 64:46-56. This effect was possibly due to the induction of specific antibody production, since *in vivo* antibody production to keyhole limpet hemocyanin (KLH) was demonstrated in many CVID patients following 3 months of PEG-IL-2 therapy. Despite these encouraging results obtained with subcutaneous PEG-IL-2, additional studies to improve immune function in CVID have not been pursued. Like parenteral IL-2, side effects including delayed type hypersensitivity

(DTH)-like reactions at the sites of subcutaneous administration and fevers, malaise, and capillary leak syndrome are seen after PEG IL-2. Since PEG-IL-2 increased half-life but resulted in increased toxicity, clinical development of PEG IL-2 has been abandoned.

EXAMPLES

Example 1 - Good Manufacturing Practice (GMP) Synthesis of IL-2 Liposomes:

Recombinant non-glycosylated human IL-2 was obtained from Chiron (Proleukin, Aldesleukin; Emeryville, CA). This preparation is FDA approved for commercial use and is provided in vials containing 1.3 mg IL-2. Contents of the vial were diluted and mixed with 1 ml sterile water. The lipid component (1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPC) is a synthetic phospholipid powder supplied by Avanti Polar Lipids (item #850345: Alabaster, AL 800-227-0651). A closed container system with a vial containing 2.5 gm of DMPC (butyl rubber stopper, room air) was used as the "reaction vessel." DMPC lipid powder was placed in these 100 mL vials with 20 mm openings (item #950000: Schott, Cleona, PA 717-228-4200). Vials were then fitted with 20 mm butyl rubber stoppers (item #1044433 S-127188 Gray: West Co, Phoenixville, PA 800-231-3000) and 20 mm flip-off seals (West Co. item #51201125).

Gamma irradiation from a cobalt source (Isomedix Services, Morton Grove, IL 847-966-1160) was used to sterilize the lipid. To ensure elimination of viable fungus (e.g. cladiosporium or aspergillus) very large doses of gamma radiation (1.3 – 1.43 Mrad-i.e. 1,300,000 cGy) were used. Pre and post radiation samples of DMPC had the same HPLC profiles.

In a laminar flow hood, 1 ml sterile water was added to each vial of IL-2 and mixed according to manufacturer's instructions (swirl gently). Then, the contents of eight IL-2 vials were added to the 100 mL vial containing 2.5 gm of sterile DMPC powder using a syringe and needle. Thus, the ratio of liquid IL-2:DMPC was 1 mg in 1 mL:300 mg. No human albumin was used as a carrier in the preparation of the IL-2 liposomes. Aluminum seals were placed over puncture holes in the septum (sterile IVA Seal, 20mm; item #CP3002; US Clinical Products, Inc., Richardson TX 214-424-5845).

The IL-2 DMPC solution in the sterile, enclosed 100 ml vial container was then subjected to vortexing, sonication, freezing, and thawing to make multilamellar liposomes

outside of the laminar flow hood without fear of contamination. The suspension was vortexed until powder was completely hydrated (2 minutes at highest speed), bath sonicated 5 minutes to create more uniform particle size and disperse aggregates, frozen in a dry ice /ethanol bath for 5 minutes, then thawed in a 37°C water bath 10 minutes for 3 cycles. After the third cycle, the IL-2 liposome concentrate was stored frozen at -20°C until future dilution and unit dose dispensing. This IL-2 liposome synthesis procedure yields a thick suspension of IL-2 liposomes that may be stored frozen for >2 years as a frozen concentrate. Dilution of the concentrate with saline and dispensing the IL-2 liposomes as a gel/liquid in unit dose vials is necessary before clinical use. Placebo liposome concentrate was made in an identical manner except 0.9% sodium chloride was added to DMPC instead of IL-2 solution.

To dilute and dispense unit doses of liposomes, 83 mL sterile 0.9% saline USP was added to one 100 mL vial of thawed liposome bulk concentrate. The diluted suspension was mixed well, warming to >25°C in water bath, if necessary. Diluted IL-2 or placebo liposomes were added to a 100 ml empty plastic iv bag (Travenol) connected to 3 way stopcock, 3 cc syringe, and extension tubing and needles. This suspension was then added in 1.1 mL unit dose portions into sterile 10 mL vials with 20 mm openings (sterile capped empty vials, item #7515ZA; Bayer, Spokane, WA 800-992-1120). Stoppers were aseptically replaced and 20 mm flip-off, tear-off seals applied (Wheaton item #224193-01 Millville, NJ 609-825-1100). Vials were placed into boxes containing 84 unit doses. Each unit dose contained 1 mL with 20 mg DMPC. IL-2 liposomes contained 1 x 10^6 IU IL-2.

Production of a lot generally took about 4 hours and required two persons for GMP standards including two-person check and sign-off of each step of the standard operating protocol. Thawing, dilution of bulk product, dispensing into unit dose vials, inspection, and labeling generally took about 3 hours. All lots passed release criteria including inspection, sterility (both bacterial and fungal culture), analysis of particle size, and assay of total IL-2 bioactivity and amount of IL-2 associated with liposome pellet. Upon dilution, an average lot of nine 100 mL vials of DMPC liposomes yielded about 1,600 1.1mL unit dose vials suitable for clinical use. As shown in Table 1, minimal lot-to-lot variation was seen for both active IL-2 containing and placebo liposomes.

In this example, DMPC was chosen as the phospholipid in the IL-2 liposome preparation because the melting temperature (Tm) is 23°C. Thus, DMPC liposomes are solid when refrigerated or nebulized at ambient room temperature and are liquid at 37°C. The latter allows *in vivo* release and IL-2:IL-2 receptor interaction. Previous experience with lipids that are solid at 37°C (e.g. dipalmitoyl phosphatidyl choline, DPPC) resulted in preparations with no IL-2 bioavailability in the IL-2 bioassay. Since the CTLL-2 bioassay of IL-2:DMPC liposomes results in similar effects as free IL-2 standard in the maintenance and proliferation of the CTLL-2 T-cell line, our IL-2 liposome formulation used in these aerosol studies was biologically active. Timed assays of diluted IL-2 liposomes in unit dose vials (data not shown) demonstrated a shelf life of approximately 12 months.

Table 1. Analysis of GMP IL-2:DMPC Liposomes and DMPC Placebo Liposomes

IL-2 Liposomes

Date of Synthesis (a,b)	Lot Number	Size (c)	IL-2 content	% IL-2 in liposomes
6/2/98; 8/25/98	MMC1-LX6	0.17	1.4	99.0
6/2/98; 9/16/98	MMC-MC8	0.11	1.3	99.0
7/21/98; 7/21/98	LK5	0.40	1.3	99.0
2/1/99; 2/3/99	MMC1-NVO	0.02	1.3	99.9
2/1/99; 2/9/99	MMC- NY1	0.01	0.83	95.7
4/12/00; 4/13/00	TDO	1.08	1.2	99.9
	Median	0.14	1.3	99.0
	Mean (s.d.)	0.30 (0.3)	1.22 (0.2)	98.8 (1.6)

Placebo liposomes

Date	Lot #	Size
6/2/98; 8/25/98	MMC1-LD8	0.02
6/2/98; 9/16/98	MMC1-MD1	0.03
11/10/98; 2/9/99	MMC-NY2	0.02

a) bulk frozen liposomes
b) diluted into unit dose vials
c) per cent particles > 15 microns by FACS analysis
d) Bioassay of total vial content of IL-2 (10^6 IU; vol 1.1mL)
% IL-2 bioactivity associated with liposome pellet after centrifugation

Example 2 – Size of IL-2 Liposomes: To assess liposome particle size and heterogeneity FACS analysis was done. Forward scattering characteristics compared to uniform latex bead standards was used to estimate size and heterogeneity. Using a Becton Dickinson FACScan apparatus equipped with an argon laser, forward light scatter (FSC) was recorded for 10,000 events /sample. Linearity of FSC was established using reference polystyrene beads (4.2 and 21.1 μm; Polysciences Inc., Warrington, PA). A histogram depicting FSC versus the number of evens was generated and compared to the FSC corresponding to the size standards. Figure 1 shows a FACS analysis estimation of liposome size distribution. Panel A is polystyrene beads of 4.2μm standard size. Panel C is polystyrene beads of 21.1μm standard size. Panels B and D are IL-2 liposomes compared to polystyrene beads of 4.2 μm standard size and polystyrene beads of 21.1 μm standard size, respectively. Note that >94% of IL-2 liposome particles being less than 4.2 μm in diameter and >99% less than 21.1 μm, as compared to standard size beads. Placebo liposome and IL-2 liposomes showed similar size distributions, with ~99% of particles being less than the size of an erythrocyte (see Table 1).

Example 3 – Bioactivity of IL-2 Liposomes: Bioactivity of the IL-2 liposomes was assessed by a thymidine incorporation assay with the IL-2-dependent CTLL-2 cell line (American Tissue Collection). Cells were maintained in RPMI media supplemented with 10% fetal calf serum (FCS) and 100 IU IL-2/mL (Chiron Corp., Emeryville, CA). The day of the assay, cells were washed four times with RPMI media and resuspended in RPMI-10% FCS at a density of $0.4x10^6$ cells/mL. Liposome samples were incubated with 20,000 IL-2-depleted CTLL-2 cells for 18 hours in 96 well microtiter plates (Co-Star, Cambridge, MA). Cells were then pulsed with 1.25 μCi of ^{3}H-thymidine (Amersham, Arlington heights, IL) for 6 h, and cell proliferation was estimated by

measuring ^{3}H-thymidine DNA incorporation in a cell harvester (Packard Instrument Company, Downers Grove, IL) after harvesting the cells onto filter paper disks (Whatman Laboratory Products, Clifton, NJ). Disks were counted in a Matrix Beta counter (Packard Instrument Company, Downers Grove, IL). The amount of bioactive IL-2 in the liposomes was calculated by comparing the counts per minute (CPM) to a standard curve generated with known amounts of free rIL-2. Results are shown in Figure 2. Figure 2A shows bioactivity of IL-2 liposomes in triplicate plates, compared to standard free rIL-2. Figure 2B shows bioactivity of the IL-2 liposome pellet compared to the supernatant, in duplicate plates.

The degree of IL-2 incorporation to the liposomes was calculated by comparing the bioactivity of the pelleted liposomes and the supernatant after centrifugation of 1 mL of a 10-fold dilution of IL-2 liposomes for 10 minutes. The percent IL-2 incorporation in the liposomes was calculated by using the dilution of the pellet (P) or the supernatant (S) required to result in 50% CPM in the following formula: [P / (P + S)] x 100. Unit dose vials were also assayed for up to 12 months to determine shelf life.

IL-2 bioactivity in the CTLL-2 bioassay demonstrated that IL-2 liposomes contained biologically active IL-2 that was approximately equally effective as the Chiron recombinant free IL-2 standard. Thus the calculated dose of IL-2 present in the liposomes closely corresponded to the assay standard in the promotion of IL-2 dependent T-cell proliferation. All lots passed the bioassay before release for clinical use. Variation in amount of IL-2 per unit dose was minimal (see Table 2) and averaged about 1.3 million IU per 1.1 mL vial. Figure 2A shows that liposomes containing IL-2 were able to support the growth of the IL-2-dependent cell line CTLL-2 in a dose dependent manner, comparable to free rIL-2. When bioactivity of the liposome pellet was compared to that of the supernatant, 99% of bioactive IL-2 was associated with the liposome pellet (see Figure 2B).

Similar results were also obtained using the non-radioactive Alamar Blue assay. HT2 cells were plated in complete medium (RPMI + 10% fetal bovine serum) at 1 x 10^{5}/ml, 100 ul per well in 96 well flat-bottom plates, after being washed 2x with PBS. IL-2 standards or IL-2 liposome suspension samples were added in 50 ul of complete medium. Cells were incubated for 24 hours at 37 degrees in 5% CO2. 50 ul of Alamar

Blue (Serotec #BUF0128) was added to each well, and samples were incubated 5 hours at 37 degrees. Samples were quantitated using a Millipore "Cytofluor" fluorescence microplate reader with 560 nm excitation and 590 nm emission settings.

Example 4 – Protocol Used for Administration of Aerosol Liposomes: Figure 1 is a diagram of the protocol used for the administration of aerosol liposomes and schedule of the tests. Dashed areas indicate the time frame when aerosol liposomes (with IL-2 or empty, in a randomized order) were given. Washout indicates the time when patients did not receive any treatment, to avoid carryover effects from the first aerosol liposomes administered. Blood tests included serum Ig, lymphocyte proliferation (to mitogens and antigens), and lymphocyte surface markers. PFT: pulmonary function tests; CXR: chest radiograph. Pneumovax was administered only to patients with specific antibody deficiencies at the indicated times.

Example 5 – Aerosolized Liposome Treatment: Patients were instructed in nebulizer use and inhalational technique by a nurse clinician familiar with pediatric asthma. First treatment was supervised; subsequent doses (total=84) were self-administered at home. Patients were instructed to add 2 squirts (2 mL) Bronkosaline to a unit dose vial of study drug and then to transfer contents to the nebulizer bowl for aerosol treatment. Liposomes contained about $1x10^6$ IU IL-2/dose. Treatments were given BID (i.e. ~2.5×10^6 IU/day) for 7 days in alternating weeks for 12 weeks. Every patient received 12 weeks of intermittent therapy with empty liposomes (placebo) or IL-2 liposomes (active drug), with 4 weeks of washout period (to avoid carryover effects) then the other treatment. The schedule of placebo liposomes-washout-active liposomes vs active liposomes-washout-placebo liposomes was randomized and double blinded by Mayo Clinic Oncology Pharmacy.

The closed container system permitted the patient to easily remove the flip-off, tear-off seal and stopper in order to easily add 2 ml saline for inhalation (e.g. bronkosaline). This arrangement was convenient and avoided patient manipulation of syringes and needles to self-administer each aerosol IL-2 liposome or placebo liposome treatment. Because of its efficient delivery and conical design, a Pari LC plus re-usable

nebulizer (Pari Respiratory Equipment, Midlothian, VA) was used with a standard air compressor (Healthdyne, Marietta, GA) for nebulization. Aerosol IL-2 liposome or placebo liposome treatments required about 12-15 minutes twice a day.

Example 6 – Treatment of CVID Patients: Clinical data describing 15 patients with primary immunodeficiency enrolled in this study is summarized in Table 2. The treatment protocol was reviewed by the Mayo Clinic Institutional Review Board (IRB16-98) and conducted under an investigator initiated IND (IND BB-7564). All immune deficiency patients or their guardians provided informed consent. Immune deficiency was previously demonstrated by at least one of the following: a) decreased serum Ig, b) decreased specific antibody deficiency in response to polysaccharide antigen, or c) decreased *in vitro* lymphocyte proliferation in response to mitogens or antigens. In the case of patient 9, who had specific antibody deficiency with normal Ig levels, the response to polysaccharide antigens was assessed 4 weeks after the administration of pneumovax, and IVIG was held for 6 weeks until the blood was drawn. None of the immune deficient subjects received systemic or topical steroids or any other form of immune suppression therapy for one month prior to the enrollment. Individuals previously receiving IVIG continued with regular IVIG infusions; these were scheduled shortly after the blood samples for the study were drawn.

At baseline (week 0), weeks 12 and 16, and end of therapy (week 28) history and physical exam were done. A chest radiogram was also obtained at baseline and if clinically indicated. Pulmonary function tests (PFT) were performed at each visit in a Breeze system (Medical Graphics Corp) without and with inhaled albuterol. Before proceeding to the next treatment phase and at the end of therapy, information about feasibility was assessed by asking the question "is this something you could do for months?" Infectious events and side effects were recorded in the medical record. According to study guidelines, if a patient had an event related to previous autoimmune disease for which corticosteroids were the treatment of choice (e.g. asthma or arthritis not responding to non-steroidal anti-inflammatory drugs), aerosol treatment was to be stopped. Side effects of individuals completing both placebo and IL-2 liposomes were compared. Pulmonary function parameters FVC and FEV1 were compared.

Ten of the 15 patients enrolled completed both active and placebo arms of the study. Five patients were withdrawn from the study; 3 of the 5 possibly had increased autoimmune disease related to treatment. These patients required systemic steroids to control worsening reactive lung disease or systemic arthritis. Two patients were withdrawn for reasons unrelated to toxicity; one developed tachycardia and edema not related to IL-2 (i.e. during a "week off" active therapy and another had lack of compliance). The ten patients that completed the study reported no significant side effects associated with either IL-2 or placebo liposome treatments. None of these patients reported any respiratory symptoms.

Serum Ig and lymphocyte surface markers did not change during the IL-2 liposome phase, compared to the placebo phase. As shown in Table 1, most patients had abnormal *in vitro* cellular responses. Lymphocyte proliferation assays to mitogens and antigens did not show significant improvement during the active drug and placebo phases, as compared to normal controls. Two patients with specific antibody deficiency with normal sIg, were repeatedly immunized with pneumovax, and the antibody response to this vaccine was assessed by measuring the antibody titers to 12 pneumococcal serotypes 4 weeks following the vaccination. Results showed that treatment with IL-2 liposomes for 12 weeks did not increase the antibody response to polysaccharide antigens.

Chest radiograms did not show any significant change in any of the patients throughout the duration of the study. Objective measures of pulmonary function (PFT) were similar pre-study, after placebo liposomes, and after IL-2 liposomes. None of the patients had any significant infections while on the study. Three patients reported subjectively feeling better while receiving the active drug, as compared to placebo and seven patients could not describe a difference in the sense of well-being between either treatment arm. In summary, treatment with the aerosol route, intermittent weekly schedule of IL-2 liposomes was safe and very well tolerated in most patients.

Patients had serum Ig, lymphocyte proliferation assay (to mitogens and antigens), and lymphocyte surface markers prior to first treatment. Studies were then repeated after the first 12 weeks of intermittent, weekly liposome treatment (week 12), after the 4 weeks of washout (week 16) and then after the 12 weeks of the second liposome treatment (week 28). In addition, patients with normal serum Ig and specific antibody deficiencies

that had shown no response to polysaccharide antigens in the past (patients 8 and 9) were immunized with pneumovax at weeks 8, 12 and 24 and specific pneumoccocal serologies were measured 4 weeks later (weeks 12, 16 and 28). Figure 3 depicts the clinical and laboratory monitoring.

Serum Ig were measured by nephelometry (Beckman Instruments Inc.). Antibody titers to twelve pneumoccocal serotypes were performed by Specialty Laboratories (Santa Monica, CA) four weeks after the administration of the pneumovax vaccine (Lederle Laboratories, Pearl River, NY) in selected patients with specific antibody deficiency (patients 8 and 9). Lymphocyte blastogenesis was assessed by measuring ^{3}H-thymidine incorporation in response to mitogens (pokeweed, phytohemagglutinine and concanavaline A from Sigma Chemicals) or antigens (Candida albicans from Bayer Allergy Products and Tetanus Toxoid from U. Massachusets Medical Center Biological Lab.). The peak lymphocyte proliferation response of each patient for each mitogen or antigen was compared with the peak lymphocyte proliferation response of a pool of normal controls. The expression of lymphocyte surface markers in blood was determined by flow cytometry with specific antibodies (CD3, CD4, CD8, CD19, CD16, CD25, HLA-DR).

Table 2. Clinical Characteristics of Patients with Primary Immune Deficiency

Patient	Age/gender	PID	Associated diseases	Rx IVIG	Ig at diagnosis (normal range mg/mL) IgA (40-500)	IgM (60-300)	IgG (700-1500)	lymphocyte proliferation mitogens	antigen
#1	54/F	CVID	Bronchiectasis Enteropathy	+	<6	29	<33	-	-C
#2	46/F	CVID	Pneumonias Bronchiectasis Sinusitis	+	<6	8.6	unknown	nl	nl
#3	10/M	CVID	Pneumonia Sinusitis	+	13	32	185	-	-C
#4	61/M	CVID	RA, thymoma Pneumonia Sinusitis renal CA Hypothyroidism	-	<6	8.4	<33	-	nl
#5	39/F	CVID	Bronchiectasis	+	85	143	500	nl	nl
#6	34/M	CVID	Bronchiectasis	+	<6	11	213	PWM -	nl
#7	55/F	CVID	Pneumonia Sinusitis	+	<6	20	110	nl	-T
#8	16/F	Ab def.	Pneumonia Craniosynostosis VonWillebrand	-	296	44	1110	-PWM, ConA	-C
#9	21/M	Ab def.	pneumonia, empyema Encephalitis	+	179	42	1290	-	-C
#10	8/M	T cell def	Sinusitis OM	-	35	29	600	-	-C
#11	13/M	CVID	Asthma Splenomegaly Lymphadenopathy	+	<6	143	350	-PWM, ConA	nl
#12	64/M	CVID	Arthritis Lymphoma	+	<6	15.6	98	-	nl
#13	3/M	T cell def	Urticaria	-	292	151	1190	-	-C
#14	29/F	CVID	lung granulomas	+	<6	62	100	-	-C
#15	27/M	CVID	Lymphoma	+	<6	9.5	392	-	nl

RA = rheumatoid arthritis; OM = otitis media
PWM = pokeweed mitogen; C = Candida; T = Tetanus.

Example 7 - Aerosol Liposomal Interleukin-2 (aIL-2) Combined with Interferon α-2b/Ribavirin Treatment in Chronic Hepatitis C: Triple therapy with aerosol IL-2 liposomes plus Interferon α-2b/Ribavirin (IFN/R) is an effective and safe therapeutic approach for chronic Hepatitis C (HCV) patients who fail standard treatment. Most chronic HCV patients, nonresponders (NR) or relapsers (Rel), fail to have viral eradication after 6-12 months therapy with IFN/R. Since retreatment with IFN/R has only limited efficacy with sustained response rates below 5%, novel therapeutic approaches are needed. IL-2 promotes proliferation and cytotoxicity of T-lymphocytes and increases T-cell responsiveness of poorly immunogenic antigens including viral proteins. Twenty-nine patients with serologic, virologic and histologic evidence of HCV were enrolled. All patients had elevated serum levels of the liver enzyme alanine transaminase (ALT) at entry and all received triple therapy for 24 weeks, followed by INF/R treatment for 24 weeks if HCV RNA was negative at 24 weeks. Therapy was discontinued for those that remained positive for HCV RNA at 24 weeks. Standard doses of IFN/R were used. A dose of 1 MU of aerosol IL-2 liposomes twice daily every other week was administered, by the same protocol outlined in Example 6, above. Sixteen of 29 patients have completed therapy (12 NR and 4 Rel). Five of 16 (31%) had sustained virologic and biochemical responses. All 4 Rel (100%) had sustained virologic and biochemical responses. Sustained responses were uncommon in NR (1/12). However, a decline in HCV RNA titer of > 1 log unite from pretreatment values was observed in 12/12 NR who failed to clear the virus. Interestingly, 3 (43%) patients who had a repeat liver biopsy after discontinuation of treatment had point decline in fibrosis stage. Two of the 3 patients with improved fibrosis stage had no sustained virologic response to triple therapy. No serious adverse events associated with the triple therapy were observed. All hepatitis C patients treated with aerosol IL-2 liposomes demonstrated a significant decrease (~10-fold) in viral RNA titer. Some patients had elimination of detectable virus; others even had reduction in cirrhosis on liver biopsy.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating a chronic viral infection comprising selecting a patient with a chronic viral infection and administering IL-2 liposomes.

2. The method of claim 1, wherein said chronic viral infection is hepatitis.

3. The method of claim 1, wherein said chronic viral infection is HIV or AIDS

4. The method of claim 2, wherein said chronic viral infection is a hepatitis B infection.

5. The method of claim 2, wherein said chronic viral infection is a hepatitis C infection.

6. The method of claim 5, wherein said IL-2 liposomes are administered by aerosol.

7. The method of claim 5, further comprising administering interferon α-2b and ribavirin (IFN/R) to said patient.

8. The method of claim 5, wherein said IL-2 liposomes are self-administered.

9. A method of treating a patient comprising administering aerosolized IL-2 liposomes, wherein said patient suffers from a condition selected from the group consisting of a bacterial infection, a viral infection, a fungal infection, a parasitic infection, and an immunodeficiency condition.

10. The method of claim 9, wherein said patient suffers from common variable immunodeficiency (CVID).

11. The method of claim 9, wherein said patient suffers from human immunodeficiency virus (HIV).

12. The method of claim 9, wherein said patient suffers from complications of AIDS.

13. The method of 9 wherein said aerosolized IL-2 liposomes are self-administered.

14. A kit comprising sterile, lyophilized IL-2 liposomes.

15. The kit of claim 14, further comprising a label or package insert indicating that said lyophilized IL-2 liposomes are to be reconstituted and administered via nebulizer treatment.

16. The kit of claim 14, wherein said IL-2 liposomes are albumin-free.

17. The kit of claim 14 further comprising an additional agent.

18. A composition comprising IL-2 liposomes.

19. The composition of claim 18, wherein said liposomes are free of albumin.

20. The composition of claim 18 further comprising an additional agent.

21. An inhaler or nebulizer comprising the composition of claim 18.

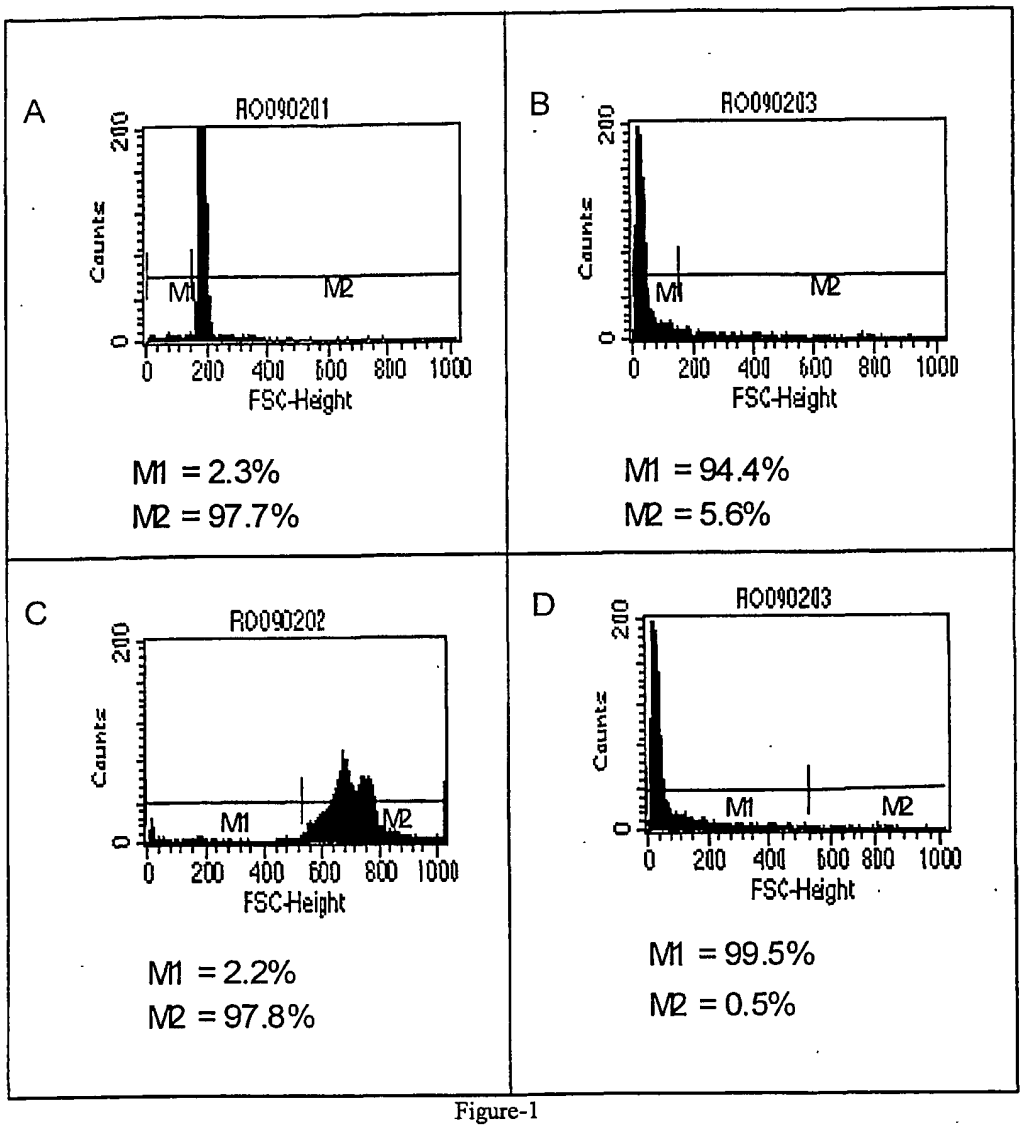
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Figure-1

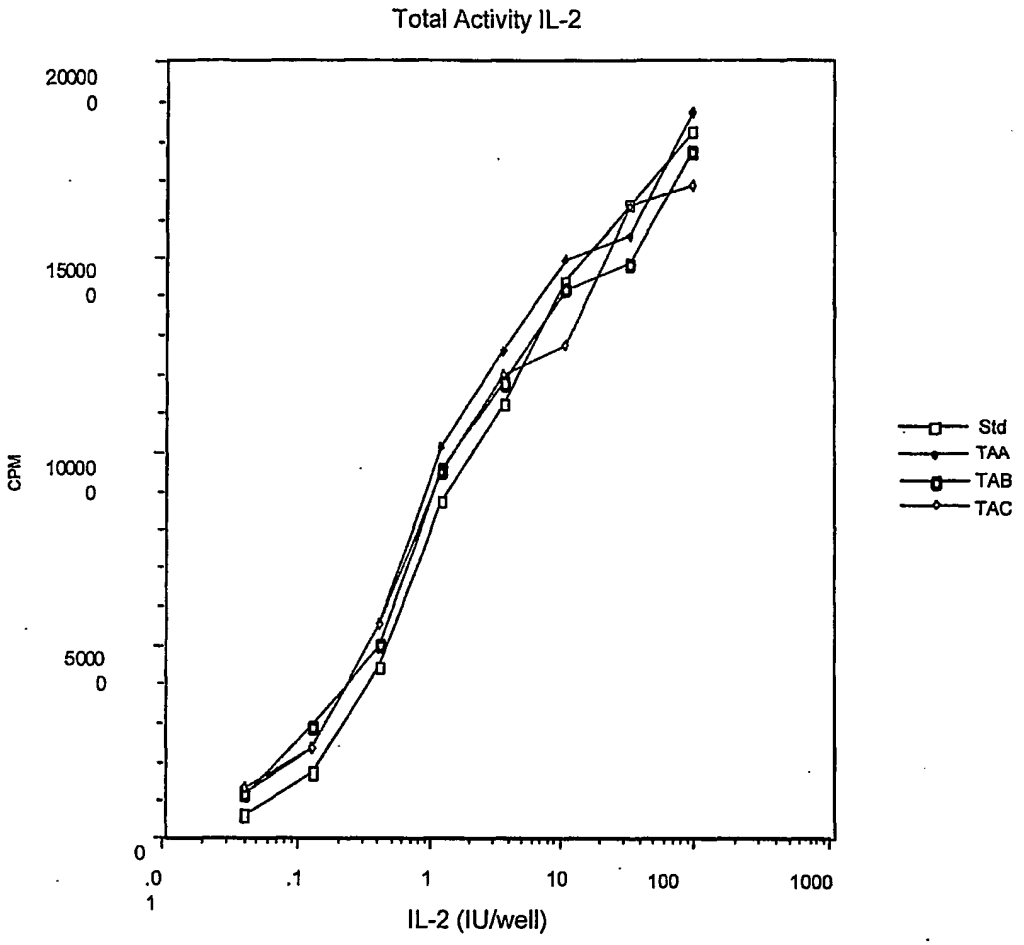
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Figure 2A

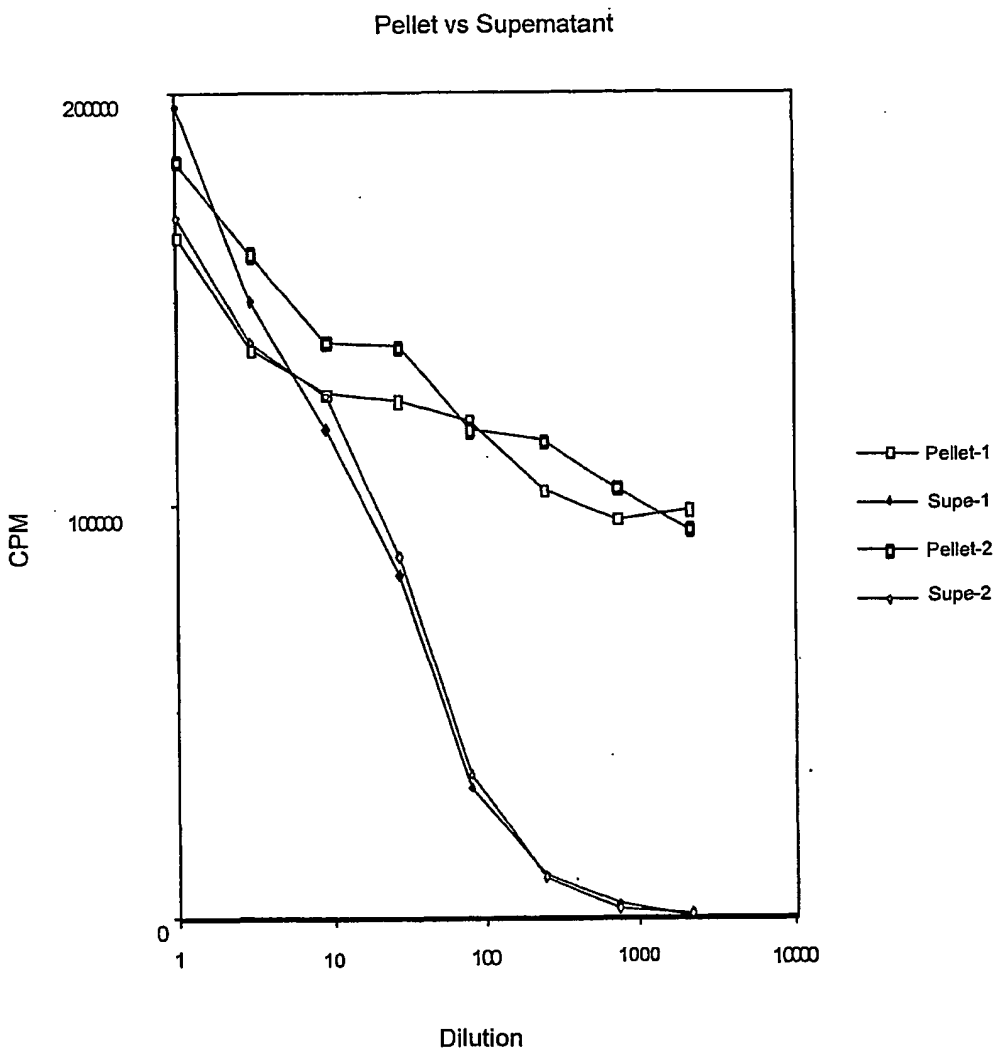
Pellet vs Supematant
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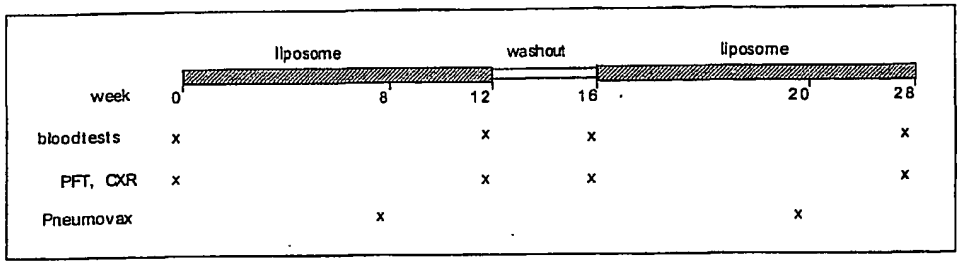
Figure 2B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20
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bloodtests
PFT, CXR
Pneumovax

Figure 3